PATENT
Customer No. 22,852
Attorney Docket No. 2956.0073-01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Sebastian SUERBAUM et al.

Serial No.: 09/015,078

Filed: January 29, 1998

For: CLONING AND CHARACTERIZATION OF THE FlbA GENE OF *H. PYLORI*, PRODUCTION OF AFFLAGELLATE STRAINS

Group Art Unit: 1647

Examiner: Sharon L. TURNER

Commissioner for Patents and Trademarks
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. § 1.132

I, Agnès Labigne, do hereby declare and say:

1. I am one of the joint inventors of the subject matter disclosed in U.S. patent application Serial No. 09/015,078.

2. Attached hereto as Exhibit A are claims 66-87.

3. On information and belief, claims 66-87 of Exhibit A are pending in U.S. patent application Serial No. 09/015,078.

4. I have examined claims 66-87 of Exhibit A and I believe that I am a joint inventor of the subject matter of these claims.

5. I am coauthor of an Abstract identified as Suerbaum et al., "Cloning, Sequencing, and Mutagenesis of the *H. pylori flbA* Gene - a Homolog of the

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lcrD/flbF/invA Family of Genes Associated with Motility and Virulence," a copy of which is attached hereto as Exhibit B.

6. I am also coauthor of an Abstract identified as Suerbaum et al., "Cloning, Expression, and Mutagenesis of the *H. pylori flbA* Gene - a Homolog of the *lcrD/flbF* Family of Genes Associated with Motility and Virulence," a copy of which is attached hereto as Exhibit C.

7. Collectively, these abstracts will hereinafter be referred to as the "Suerbaum Abstracts."

8. I am a joint inventor on the Suerbaum Abstracts.

9. On information and belief, the Suerbaum Abstracts have been cited against claims 66-78 in the U.S. patent application Serial No. 09/015,078 because A. Schmitz and C. Josenhans are named as coauthors of the Suerbaum Abstracts, but are not named as coinventors in U.S. patent application Serial No. 09/015,078, and based on these circumstances, the U.S. Patent Examiner asserted that the subject matter disclosed in U.S. patent application Serial No. 09/015,078, and the subject matter claimed in claims 66-87 in Exhibit A, were not invented by the inventors named in the application.

9. The experimental work described in the Suerbaum Abstracts was conducted by Sebastian Suerbaum, or Agnès Labigne, or performed under our direction or supervision.

10. A. Schmitz and C. Josenhans worked under our supervision. Under our instructions, A. Schmitz rendered technical analysis in sequence analysis and C. Josenhans rendered technical assistance with immunoblot experiments. A. Schmitz

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and C. Josenhans were rewarded for their work by being included as coauthors of the Suerbaum Abstracts.

11. A. Schmitz and C. Josenhans did not make an inventive contribution to the experimental work described in the Suerbaum Abstracts, or an inventive contribution to the subject matter disclosed in U.S. patent application Serial No. 09/015,078, or an inventive contribution to the subject matter of claims 66-87 of Exhibit A, and A. Schmitz and C. Josenhans are not joint inventors of this subject matter.

12. The undersigned declares further that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issuing therefrom.

Signed at Paris, France By: _____
Agnès Labigne

Dated: September 16, 2003

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66. An *H. pylori* bacterial strain, or an extract of an *H. pylori* bacterial strain, wherein the *H. pylori* bacterial strain has an aflagellate phenotype resulting from a mutation in the *flbA* gene of the *H. pylori* bacterial strain.

67. The *H. pylori* bacterial strain, or extract of an *H. pylori* bacterial strain, according to claim 66, wherein the *flbA* gene is able to hybridize, under conditions of stringency, with a probe corresponding to a nucleotide fragment from *H. pylori*, which has been amplified using two oligonucleotides having the following sequences:

OLF1bA-1: ATGCCTCGAGGTCGAAAAGCAAGATG (SEQ ID NO:1),

OLF1bA-2: GAAATCTTCATACTGGCAGCTCCAGTC (SEQ ID NO:2).

68. The *H. pylori* bacterial strain, or extract of an *H. pylori* bacterial strain, according to claim 66, wherein the *flbA* gene comprises SEQ ID NO: 6.

69. The *H. pylori* bacterial strain, or extract of an *H. pylori* bacterial strain, according to claim 66, wherein the *H. pylori* bacterial strain does not express the hook protein (or anchoring protein) of the flagellum of *H. pylori*.

70. The *H. pylori* bacterial strain, or extract of an *H. pylori* bacterial strain, according to claim 66, wherein the *H. pylori* bacterial strain also lacks the flagellum sheath.

71. The *H. pylori* bacterial strain, or extract of an *H. pylori* bacterial strain, according to claim 70, wherein the *H. pylori* bacterial strain does not express the hook protein (or anchoring protein) of the flagellum of *H. pylori*.

72. The *H. pylori* bacterial strain, or extract of an *H. pylori* bacterial strain, according to claim 66, wherein the *H. pylori* bacterial strain is obtained from strain N6 having deposit Accession No. NCIMB 40512.

73. The *H. pylori* bacterial strain, or extract of an *H. pylori* bacterial strain, according to claim 66, wherein the *H. pylori* bacterial strain is strain N6flbA$^-$ having deposit Accession No. NCIMB 40747.

74. The extract of an *H. pylori* bacterial strain according to claim 66, wherein the bacterial extract is a total bacterial extract.

75. The extract of an *H. pylori* bacterial strain according to claim 66, wherein the bacterial extract is a n-octyl glucoside extract.

76. The extract of an *H. pylori* bacterial strain according to claim 66, wherein the bacterial extract is obtained after extracting with PBS or glycine.

77. An *H. pylori* bacterial strain, or an extract of an *H. pylori* bacterial strain, wherein the *H. pylori* bacterial strain has an aflagellate phenotype resulting from a mutation in the *flbA* gene of the *H. pylori* bacterial strain, and wherein the *H. pylori* bacterial strain does not express the FlaA and FlaB proteins.

78. The *H. pylori* bacterial strain, or extract of an *H. pylori* bacterial strain, according to claim 77, wherein the *flbA* gene is able to hybridize, under conditions of stringency, with a probe corresponding to a nucleotide fragment from *H. pylori*, which has been amplified using two oligonucleotides having the following sequences:

OLF1bA-1: ATGCCTCGAGGTCGAAAAGCAAGATG (SEQ ID NO:1),

OLF1bA-2: GAAATCTTCATACTGGCAGCTCCAGTC (SEQ ID NO:2).

79. The *H. pylori* bacterial strain, or extract of an *H. pylori* bacterial strain, according to claim 77, wherein the *flbA* gene comprises SEQ ID NO: 6.

80. The *H. pylori* bacterial strain, or extract of an *H. pylori* bacterial strain, according to claim 77, wherein the *H. pylori* bacterial strain does not express the hook protein (or anchoring protein) of the flagellum of *H. pylori*.

81. The *H. pylori* bacterial strain, or extract of an *H. pylori* bacterial strain, according to claim 77, wherein the *H. pylori* bacterial strain also lacks the flagellum sheath.

82. The *H. pylori* bacterial strain, or extract of an *H. pylori* bacterial strain, according to claim 81, wherein the *H. pylori* bacterial strain does not express the hook protein (or anchoring protein) of the flagellum of *H. pylori*.

83. The *H. pylori* bacterial strain, or extract of an *H. pylori* bacterial strain, according to claim 77, wherein the *H. pylori* bacterial strain is obtained from strain N6 having deposit Accession No. NCIMB 40512.

84. The *H. pylori* bacterial strain, or extract of an *H. pylori* bacterial strain, according to claim 77, wherein the *H. pylori* bacterial strain is strain N6flbA$^-$ having deposit Accession No. NCIMB 40747.

85. The extract of an *H. pylori* bacterial strain according to claim 77, wherein the bacterial extract is a total bacterial extract.

86. The extract of an *H. pylori* bacterial strain according to claim 77, wherein the bacterial extract is a n-octyl glucoside extract.

87. The extract of an *H. pylori* bacterial strain according to claim 77, wherein the bacterial extract is obtained after extracting with PBS or glycine.

Cloning, Sequencing, and Mutagenesis of the *H. pylori flbA* Gene - a Homolog of the *lcrD/flbF/invA* Family of Genes Associated with Motility and Virulence.

S. Suerbaum, A. Schmitz, C. Josenhans. A. Labigne
Med. Microbiol., Ruhr-University Bochum (Germany), and Unité des Entérobactéries, INSERM U389, Institut Pasteur, Paris (France).

The HP flagellar filament contains two functionally different flagellin molecules, FlaA and FlaB. The genes encoding these flagellins have been cloned and characterized. The ability of the bacterium to regulate the amounts of FlaA and FlaB in the filament is believed to play a role in the adaptation of the filament to environmental conditions (viscosity), however, little is known about motility regulation in HP. Recently, a family of conserved proteins involved in the regulation and/or secretion of virulence-associated proteins has been described. Two members of this family, *Caulobacter crescentus* FlbF and *Campylobacter jejuni* FlbA are required for flagellar biosynthesis and considered regulators of motility intervening at the beginning of the regulatory cascade of motility proteins. We have cloned the gene coding for the LcrD/FlbF homolog of HP. A fragment of the gene was amplified from the HP chromosome using degenerate primers based on highly conserved regions of the proteins and then used to screen a HP cosmid gene bank. After subcloning, the gene (2.1 kb, designated *flbA*) was sequenced. The predicted FlbA gene product had a predicted molecular weight of 79 kDa and exhibited high degrees of homology with the other known members of the LcrD/FlbF protein family. The open reading frame was preceded by a putative sigma 28 consensus promoter sequence. Isogenic mutants of *H. pylori* in the *flbA* gene were constructed by disruption with a kanamycin resistance cassette and electroporation-mediated allelic exchange. The *flbA* mutants were characterized by SDS-PAGE, Western blotting, motility testing and electron microscopy. FlbA mutants were completely devoid of flagella and did not express FlaA or FlaB protein. In concordance with these data, *flbA* mutants were completely non-motile. In addition, *flbA* mutants lacked several other protein bands that may represent yet unknown virulence-associated or flagellar proteins and that are presently being characterized.

186

EXTRACT OF *HELICOBACTER PYLORI* INDUCES GASTRIC MICROCIRCULATORY DAMAGE IN RATS
-ROLE OF ACTIVATED NEUTROPHILS-

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Dept. of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan

H.pylori-derived chemotaxin has been proposed as one of virulent factors in gastritis and gastric ulcer. In this paper, the influence of water extract of *H.pylori* in rat mesenteric and gastric microcirculation was investigated by using intravital microscopy

ABSTRACTS

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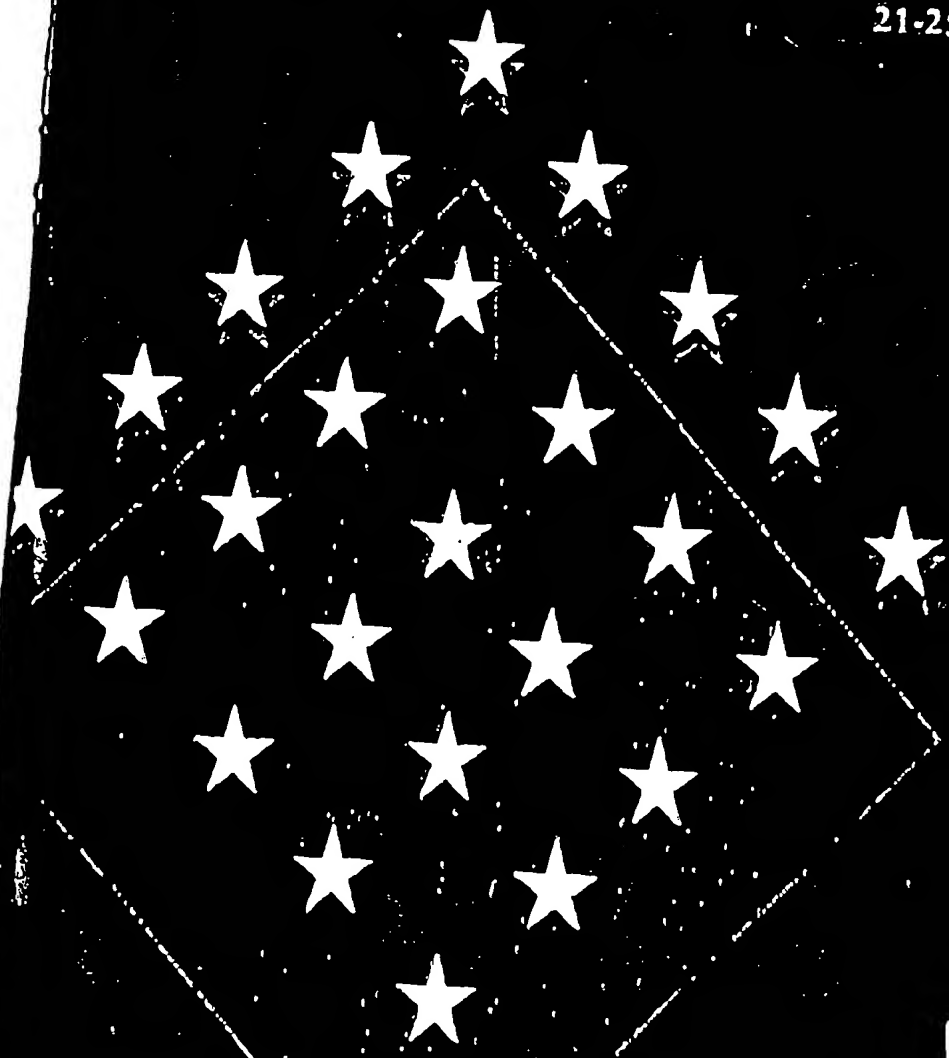

ISSN 0067-2777

B-89 The Role of Chemotaxis in Invasion of *Campylobacter jejuni* into Eukaryotic Cells. RUIJIN YAO*, DON H. BURR AND PATRICIA GUERRY. Enteric Diseases Program, Naval Medical Research Institute, Bethesda, MD and the FDA, Washington, D. C.

Motility has been shown to be an important virulence determinant for *Campylobacter* spp. Non-flagellated and flagellated but non-motile (paralyzed) organisms are unable to colonize in vivo or to invade epithelial cells in vitro. In order to determine if chemotaxis or motility itself is necessary, we have examined the role of the *cheY* gene of *C. jejuni* in in vitro invasion. The *cheY* gene of *C. jejuni* 81-176 was cloned and sequenced and shown to encode a predicted protein of 149 amino acids with a predicted M_r of 13,191. The predicted protein shares 40% identity and 67% similarity with the CheY protein of *Salmonella typhimurium*. The mutant was isolated by insertion of a kanamycin resistance cassette within the ORF, and reintroduction of the mutated allele into 81-176 by natural transformation. The resulting mutant was motile when examined by wet mount, but non-chemotactic on motility agar and in chemotactic assays. In in vitro invasion assays using INT407 cells, the *cheY* mutant invaded as well or slightly better than the parental strain, indicating that loss of chemotaxis does not significantly alter the invasiveness of *C. jejuni* in this assay. The role of chemotaxis in vivo is under investigation.

B-90 *cagII*, a New Multigene Locus Only Present in the Most Virulent *Helicobacter pylori* Strains N. S. AKOPYANTS, D. KERSULYTE and D. E. BERG* Dept. Molec Microbiol, Washington Univ. Med Sch., St. Louis, MO 63110

Helicobacter pylori strains that lead to peptic ulcers and gastric cancer produce a vacuolating cytotoxin (*vacA*) and an immunodominant cytotoxin-associated protein (*cagA*), whereas many strains from asymptomatic carriers do not produce either protein. We searched for new genes associated with *H. pylori* virulence using our ordered library of *H. pylori* cosmid clones (*1*) by hybridizing restriction digests of each cosmid with DNA from several *cagA*- (non-vir) and control *cagA*+ (vir) strains. A ~20 kb segment termed "*cagII*" was found in *cagA*+ strains, but was absent from *cagA*- strains. This *cagII* segment was cloned and, in turn, used as a hybridization probe. It hybridized with DNAs from each of 73 *cagA*+ strains, but not with DNAs from 32 of 33 *cagA*- strains. The *cagII* segment is hundreds of kb from *cagA* and from *vacA*. Assuming interstrain recombination during mixed infection (see 2) akin to that seen in the laboratory, we propose that the epidemiologic association between *cagII*, *cagA* and *vacA* is due to selection for functions they encode, not to chromosomal linkage. The ends of the *cagII* segment are being sequenced to gain insights into how *cagII* is acquired or lost during evolution. In addition, we are sequencing the entire 22 kb *cagII* region (collaboration with S. Clifton and B. Roe), in the expectation that some *cagII* encoded proteins will affect host interactions during *H. pylori* infection -- e.g., mucosal immune response, or extent of tissue damage or cell proliferation.

REFERENCES 1. Bukanov & Berg. Molec. Micro. 11:509-523, 1994.
2. Akopyants, Eaton & Berg Inf. Immun. in press (Jan. 1995).

B-91

Colonization of Gnotobiotic Piglets by *H. pylori* Deficient in Two Flagellin Genes K. EATON*, S. SUERBAUM, C. JOSENHANS, S. KRAKOWKA: Ohio State University, Columbus, OH USA and Ruhr-Universitat Bochum, Bochum, Germany

H. pylori possesses two flagellin molecules, FlaA, the major species, and FlaB, which is expressed in minor amounts, at least *in vitro*. Isogenic mutants of *H. pylori*, strain N6, were constructed by disruption of the *flaA* or *flaB* genes with a kanamycin (*km*) resistance cassette, or by introduction of both *km* and a chloramphenicol resistance gene (*cat*) to produce a double mutant.

This study sought to determine if one or both flagellin species are necessary for colonization or persistence by H. pylori. Eighteen gnotobiotic piglets were given one of 4 isogenic strains of *H. pylori* orally, and killed 2 or 4 days post-inoculation (PI). Strain N6, the wild-type, produced both FlaA and FlaB and was fully motile in 0.5% agar. N6*flaA*::*km* expressed FlaB, but not FlaA, produced short, truncated flagella, and was weakly motile. N6*flaB*::*km* produced FlaA, but not FlaB, had apparently normal flagella, and was moderately motile, and N6*flaA*::*cat*/*flaB*::*km* did not produce any flagella and was non-motile.

Wild-type strain N6 colonized all piglets at both time intervals (mean *cfu*/g gastric mucosa=8.9x10^6). Both N6*flaA*::*km* and N6*flaB*::*km* also colonized at both time points, but colonization was weak (50-500*cfu*/g). In addition, in contrast to urease-negative mutants which fail to persist *in vivo*, colonization by both flagellin-negative mutants increased approximately one order of magnitude between 2 and 4 days PI. The double mutant, N6*flaA*::*km*/*flaB*::*cat* also colonized for two days PI, but not 4 days PI.

These findings demonstrate that both flagellin species are necessary for full colonization by *H. pylori*, in spite of the fact that FlaB apparently has a minor role in motility *in vitro*. Weak colonization and persistence is possible in the absence of either flagellin species, but not both. Future studies will determine the duration of persistence of flagellin-negative strains.

B-92

Characterization of a Cell Proliferation Inhibiting Factor (PIF) Produced by *Helicobacter pylori*. U. KNIPP*, W. KAUP, S. BIRKHOLZ, and W. OPFERKUCH. Ruhr-Univ. Bochum, Bochum, FRG.

Previous examinations by our group on *Helicobacter pylori* (HP), the causative agent of chronical type B gastritis in man, showed that a cytoplasmic fraction (CF) of HP suppresses the in vitro proliferative response of human mononuclear cells to mitogens and antigens. The present study demonstrates that the antiproliferative activity of CF also affects the spontaneous proliferation of different mammalian cell lines including U937, Jurkat and Kato3. This effect was obvious in the first 16 hours of incubation and maximal between 24 to 48 hours. In addition CF significantly diminished the protein synthesis of the cells in the first 6 hours of incubation comparable to cycloheximide and diphtheria toxin. The urease and the vacuolizing cytotoxin of HP could be excluded as the causative agents for the antiproliferative effect using an isogenic urease negative mutant strain and cytotoxin negative strains. So far the inhibitory effects were not due to lytic or other lethal activities of CF. A preliminary physico-chemical characterization showed that the proliferation inhibiting factor (PIF) was non-dialyzable, heat-labile (70 °C, 30 min), sensitive to proteases and had an apparent native molecular weight of 100±10 kDa. These results implicate the presence of a protein factor in HP with antiproliferative activity for immunocompetent and epithelial mammalian cells. It is reasonable to presume that this property may contribute to the pathogenesis of HP induced diseases.

B-93

Cloning, Expression, and Mutagenesis of the *H. pylori flbA* Gene - a Homolog of the *lcrD/fliF* Family of Genes Associated with Motility and Virulence. S. SUERBAUM[1,*], A. SCHMITZ[1], C. JOSENHANS[1], A. LABIGNE[2]
[1]Med. Microbiol. & Immunol., Ruhr-University, Bochum (Germany), and [2]Unité des Entérobactéries, INSERM U389, Institut Pasteur, Paris (France)

Motility is an important virulence factor of *Helicobacter pylori* (HP). The genes encoding the flagellin proteins FlaA and FlaB have been cloned, but little is known about the regulation of flagellar biogenesis in this organism. Recently, a family of conserved proteins involved in the regulation and/or secretion of virulence-associated proteins has been described. Several members of this family are required for flagellar biosynthesis and considered regulators of motility intervening at the beginning of the regulatory cascade of motility proteins. We have cloned the gene coding for the LcrD/FliF homolog of HP. A fragment of the gene was amplified from the HP chromosome using degenerate primers and then used to screen a HP cosmid gene bank. After subcloning, the gene (2.2 kb, designated *flbA*) was sequenced. The predicted FlbA gene product had a molecular mass of 80.1 kDa and exhibited high degrees of homology with the other known members of the LcrD/FliF protein family. The *flbA* gene was PCR-cloned into the *E. coli* expression vector pQE30. Purification studies with the recombinant protein provided evidence that the protein has strong affinity for membranes, consistent with the strong hydrophobicity of the N-terminal part of FlbA. Isogenic mutants of HP in the *flbA* gene were constructed by disruption with a kanamycin resistance cassette. The *flbA* mutants were characterized by Western blotting, motility testing, and electron microscopy. *FlbA* mutants were completely devoid of flagella and flagellar sheaths and did not express FlaA or FlaB protein. In concordance with these data, *flbA* mutants were completely non-motile. In addition, *flbA* mutants lacked several other protein bands that may represent yet unknown virulence-associated or flagellar proteins.

B-94 *In Vitro* Translocation of *Helicobacter pylori* through Epithelial Cells. LUQUEÑO-BAZTENET V.*, and RUIZ-PALACIOS GM. Nat. Inst. Nutrition. Mexico.

Adherence of *Helicobacter pylori* to epithelial cells is needed to establish the interaction between bacteria and the gastric mucosa, although other features, such as hydrophobicity and production of urease and cytotoxin (Ctx), may play a role in this process. To study the bacteria-mucosa interaction by adherence and transcytosis through polarized cells, 3 prototype strains from different clinical presentations were studied. Sources of strains were: 1 Ctx$^+$ from chronic gastritis (Mexico), and 2 Ctx$^+$ from duodenal ulcer (USA and U.K.). Adherence was studied by the HEp-2 cell assay; transcytosis was assessed by counting the number of viable bacteria able to pass through a monolayer of polarized Caco-2 cells; and the monolayer integrity by measuring transepithelial resistance and by electron microscopy.

All strains were cell-adherent. Two adherence patterns were identified by the HEp-2 cell assay: diffuse (2str.) and aggregative (1 str). The most significant finding was that *H. pylori* strains were able to translocate within 8 h across polarized Caco-2 cells without disrupting the monolayer. There was no correlation between clinical presentation of *H. pylori* infection and cell adherence or transcytosis across the polarized cell monolayer.
The ability of *H. pylori* to translocate without damaging epithelial cells seems to be an inherent property of the bacteria, and may be an important step towards reaching the lamina propria and induce an inflammatory response.